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Enhanced efficacy of 7-hydroxy-3-methoxycadalene via glycosylation in in vivo xenograft study

Hyang Yeon Lee,[a,†] Jung-Taek Kwon,[b,†] Minseob Koh,[a] Myung-Haing Cho[b,*] and Seung Bum Park[a,*]

[a]*Department of Chemistry, Seoul National University, Seoul 151-747, Republic of Korea*
[b]*College of Veterinary Medicine, Seoul National University, Seoul 151-742, Republic of Korea*

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Abstract—7-Hydroxy-3-methoxycadalene, isolated from *Zelkova serrata* Makino, was confirmed as a biologically active natural compound. In this study, the efficacy of cadalene as an anticancer agent was tested. In order to address the poor physicochemical properties of cadalene, we designed and synthesized glycosylated cadalene derivatives for improved solubility and efficient drug delivery as a potential prodrug. In vitro cell viability assays confirmed that glycosylated cadalenes were less toxic and more soluble than cadalene. In an in vivo xenograft study in mice, the oral administration of glycosylated cadalenes caused a significant reduction in tumor size.

Cadalene (7-hydroxy-3-methoxycadalene, **1**), a small molecular secondary metabolite classified as a plant-derived flavonoid,[1] was isolated from *Zelkova serrata* Makino and made available in large quantities from *Zelkova* wood powder by extraction with ethanol and purification with flash silica column chromatography. In our previous study, cadalene demonstrated various biological activities such as chemopreventive effects and antioxidative activities in murine lung tumorigenesis induced by 4-(methylinitrosamino)-1-(3-pyridyl)-1-butanone (NNK); NNK is the major causative factor of lung cancer.[2,3] When A/J mice with oxidative damages induced by NNK were orally treated with cadalene on a dosage of 100 mg/kg, the glutathione (GSH) level was maintained and the oxidative stress was reduced. With regard to its chemopreventive efficacy, cadalene-treated A/J mice (oral dosage of 100 mg/kg) demonstrated a significant reduction in the incidence of lung adenomas from 45% to 10%.[2b] The results supported that cadalene is an antioxidant and potent chemopreventive agent against NNK-induced murine lung tumorigenesis.[2] However, the efficacy of cadalene as an anticancer agent has not been studied. Furthermore, there are potential problems in the clinical application of cadalene due to its low solubility in water.

In order to address these issues, we performed the chemical modification of cadalene. Glycosylation is one of the major approaches for increasing water solubility and cellular uptake.[4–9] There are many literature precedents for the enhancement in the solubility and efficacy of natural products such as quercetin,[6] kaempferitrin,[7] and chrysoeriol.[8] Therefore, it is expected that glycosylated cadalenes will have enhanced water solubility and improved cellular uptake.[5,7] Due to the fast proliferation of cancer cells, glucose uptake in them is significantly faster than that in normal cells. [9] We also considered the importance of the stereochemistries of glycolytic linkage at anomeric positions because we observed the differences in the cellular uptakes of fluorescent glucose bioprobes in α-anomer and β-anomer.[9]

In this study, we focused on the enhancement of the water solubility and efficient delivery of cadalene via glycosylation. The transient modification of cadalene might facilitate its targeted delivery into cancer cells in the form of an inactive prodrug, which can be unmasked by the cellular enzymatic reaction of glycosidase or simple hydrolysis.[10] The antitumor efficacies of cadalene **1**

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* Corresponding authors. Tel.: +82 2 880 2376; fax: +82 2 873 1268 (M.-H.C.); tel.: +82 2 880 9090; fax: +82 2 884 4025 (S.B.P.); e-mail addresses: mchotox@snu.ac.kr; sbpark@snu.ac.kr
† These authors contributed equally to this work.

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and glycosylated cadalenes (**2** and **3**) were examined by an in vitro cell viability assay and confirmed with a human nonsmall cell lung cancer xenograft model by oral administration.

As shown in Figure 1, we designed and synthesized two derivatives of glycosylated cadalene; one derivative was obtained by direct β-glycosylation on cadalene (β-Glc-*O*-cadalene, **2**), and the other was obtained by α-glycosylation on cadalene via an ethoxy linker (α-Glc-OCH_2CH_2-*O*-cadalene, **3**). The key features of these two glycosylated cadalenes are two different stereochemistry of the hydrolytic linkage at anomeric positions, namely, α- and β-anomers. The synthesis of the glycosylated cadalene derivatives (**2** and **3**) is shown in Schemes 1 and 2. The synthetic scheme was initiated with the global acetylation of hydroxyl groups on glucose. Subsequently, the hydroxyl group at the anomeric position on compound **4** was selectively deprotected with ethylenediamine, followed by activation with trichloroacetonitrile in the presence of DBU.[11,12a] Glycosyl trichloroacetimidate **5** was a thermodynamically favored α-anomeric product, which was confirmed by ^{1}H NMR; therefore, the treatment of cadalene with glycosyl trichloroacetimidate led to the desired β-glycosylated cadalene in high yield with the assistance of BF_3•OEt_2.[12] The other derivative was α-glycosylated cadalene with an ethoxy linker, **3**. The stereochemical enrichment of the α-anomer of glycosylated cadalene was achieved by the following pathway. The treatment of glucose with 2-bromoethanol in the presence of acidic ion exchange resin yielded an α-anomer compound as the major product due to its anomeric effect.[9] After global acetylation, the α-anomer of glycosyl bromide **6** was purified by flash silica column chromatography and treated with cadalene **1** in the presence of Cs_2CO_3.[13] The deacetylation of the resulting substitution products yielded the desired α-Glc-OCH_2CH_2-*O*-cadalene, **3**.

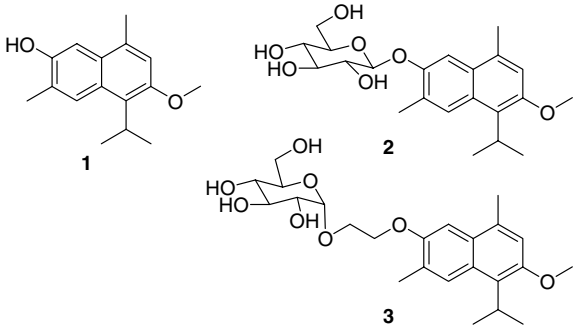
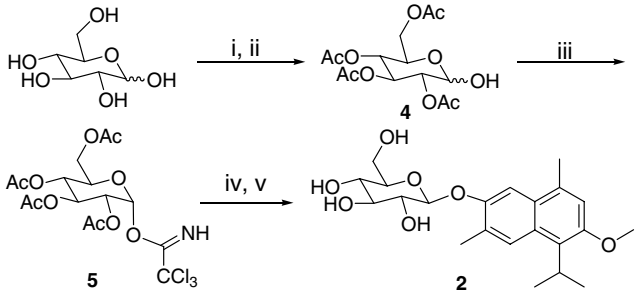
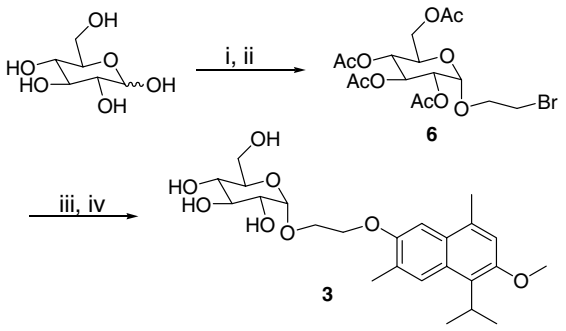


Figure 1. Structures of cadalene and glycosylated derivatives: (**1**) 7-hydroxy-3-methoxycadalene, (**2**) β-Glc-*O*-cadalene, and (**3**) α-Glc-OCH_2CH_2-*O*-cadalene.



Scheme 1. Synthesis of β-Glc-*O*-cadalene (**2**). Reagents: (i) acetic anhydride, pyridine, DMAP; (ii) ethylenediamine, glacial acetic acid, DCM, 80% from D-glucose; (iii) Cl_3CCN, DBU, DCM, 72%; (iv) **1**, BF_3·OEt_2, anhyd DMF, 89%; (v) NaOMe, MeOH, quantitative yield.



Scheme 2. Synthesis of α-Glc-OCH_2CH_2-*O*-cadalene (**3**). Reagents and conditions: (i) 2-bromoethanol, Dowex 50wx8-400 ion exchange resin, 70 °C, 82%; (ii) glacial acetic anhydride, pyridine, DMAP, 65%; (iii) **1**, Cs_2CO_3, anhyd DMF, 60 °C, 70%; (iv) NaOMe, MeOH, quantitative yield.

To test the in vitro cytotoxicity of cadalene and glycosylated cadalene, the cell viability assay was performed. Cadalene (**1**), β-Glc-*O*-cadalene (**2**), and α-Glc-OCH_2CH_2-*O*-cadalene (**3**) at indicated concentrations were treated on A549 cells (human lung carcinoma) and HeLa cells (human cervical carcinoma). The in vitro cell viability of individual compounds was measured after 24 h of treatment using a cell counting kit (Dojindo Laboratories, Kumamoto, Japan), which counts live cells with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-8) and 1-methoxyphenazine methosulfate (1-methoxy-PMS).[14] The transformation of WST-8 to formazan by metabolically active cells was quantified by the ELISA plate reader (ELx800, BioTek, USA) at a wavelength of 450 nm due to the enhanced absorbance by formazan. As shown in Figure 2, the IC_{50} values of cadalene **1**, β-Glc-*O*-cadalene **2**, and α-Glc-OCH_2CH_2-*O*-cadalene **3** on A549 cells were 34.9, 193.7, and 129.4 μM, respectively. In the case of HeLa cells, the IC_{50} values of **1**, **2**, and **3** were 28.3, 230.9, and 160.6 μM, respectively. As expected, we observed a significant reduction in the cytotoxicity of the cells and the improved solubility of glycosylated cadalene. The cell viability data on two different cancer cell lines show similar patterns of cytotoxicity reduction.

From the in vitro cell viability assay data, we could confirm that glycosylated cadalenes (**2** and **3**) have lower cytotoxicity than cadalene (**1**). In our previous research, we confirmed the chemopreventive efficacy of cadalene against the NNK-induced pulmonary tumorigenesis in mice.[2b] However, the antitumor activity of cadalene was not tested. Therefore, we performed an in vivo tumor xenograft study and tested two glycosylated

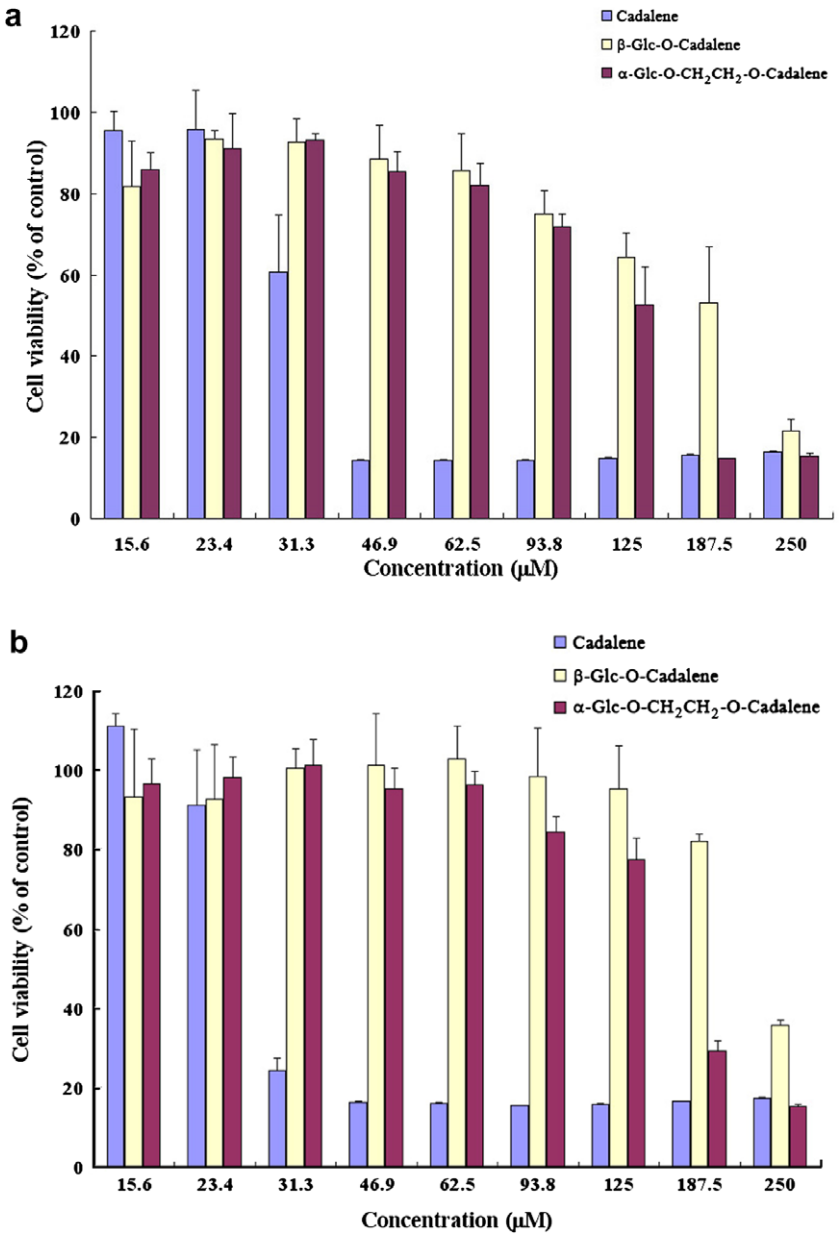


Figure 2. Cell viability data at various dosages of cadalene (**1**) and glycosylated cadalenes (**2** and **3**): (a) A549 human lung carcinoma cell line; (b) HeLa human cervical carcinoma cell line.

cadalene as prodrugs in order to achieve better solubility and enhanced drug delivery toward cancer cells (see Fig. 3). Athymic (BALB/c, nu/nu, 5 weeks old) male nude mice were obtained from SLC Inc. (Hamamatsu, Japan). The mice were housed in autoclaved plastic filter-top cages, maintained in a laboratory animal facility (23 ± 2 °C, 50 ± 20% relative humidity, 12-h light/dark cycle), and acclimatized for at least 1 week prior to the beginning of the study. All animal experiments were performed in accordance with the guidelines of Seoul National University for the care and use of animals. Xenografts were established from the A549 cell lines by the subcutaneous implantation of 1×10^7 cells in serum-free media in the flank of the animals.[15] The tumor volume was calculated by using the mean diameter measured with calipers using the formula [$v = 0.5 \times a \times b^2$],

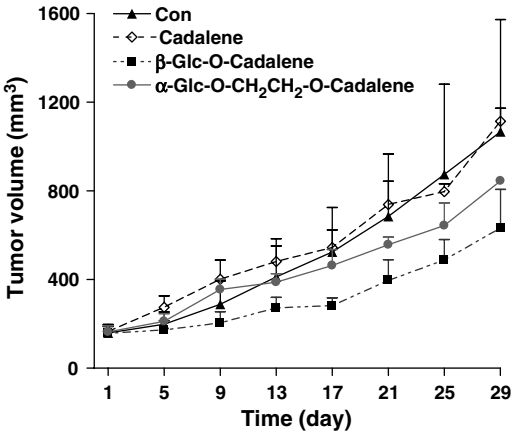


Figure 3. Xenograft data of tumor volume for 29 days.

where *a* and *b* are the largest and smallest tumor diameters, respectively.[16] Cadalene and glycosylated cadalenes were administered after the tumors grew to a size of approximately 160 mm^3 (approximately 4 weeks after implantation). The vehicle control and chemicals were administered at 100 mg/kg via oral gavage (in a mixture of 20:1 vegetable oil/DMSO) once every 28 days. After the experimental period (28 days), the tumor volume of the control increased sevenfold. Interestingly, the treatment of cadalene **1** did not affect the tumor volume. However, the oral treatment of both glycosylated cadalene derivatives (**2** and **3**) caused a significant reduction in the tumor volume. As shown in Figure 3, there was a 50% reduction in the tumor size as compared to the tumor volume of the control by the treatment of β-Glc-*O*-cadalene (**2**). In the case of α-Glc-OCH_2CH_2-*O*-cadalene (**3**), there was a 25% reduction in the tumor volume, which is consistent with the in vitro cell viability study. Therefore, we could conclude that the hydrolytic glycosylation of bioactive cadalene significantly enhances the efficacy of the anticancer agent through efficient drug delivery and improved water solubility.

In summary, cadalene is a biologically active natural product isolated from *Z. serrata* Makino. In our previous study, the biological activities of cadalene were confirmed in chemopreventive effects and antioxidative activities in NNK-induced lung tumorigenesis in mice. In this study, cadalene **1** was tested as an anticancer agent and the chemical modification of cadalene was performed in order to address the limitations of cadalene as a therapeutic agent due to its poor solubility. Glycosylation is one of the general approaches for achieving enhanced water solubility and targeted drug delivery toward cancer cells; therefore, the transient glycosylation of cadalene might facilitate its targeted delivery into the cancer cells in the form of an inactive prodrug, which can be unmasked by the cellular enzymatic reaction of glycosidase or simple hydrolysis. In this study, we synthesized two types of glycosylated cadalene (**2** and **3**) with two distinct stereochemistry of the hydrolytic linkage at anomeric positions, i.e., α- and β-anomers. The in vitro cell viability test of glycosylated cadalenes showed a significant reduction in cytotoxicity via two different cancer cell lines. The efficacy of these compounds was confirmed by an in vivo xenograft study. The oral administration of β-Glc-*O*-cadalene (**2**) induced a 50% reduction in the tumor size. Therefore, we concluded that the glycosylation of bioactive small molecules with undesirable physical properties is an efficient approach to enhance cell permeability, water solubility, and targeted drug delivery.

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Supplementary data

Supplementary data, including experimental procedures and analytical data, associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.08.071.

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